Vol. 142, No. 1 February, 1972

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

STRUCTURAL AND CHEMICAL ASPECTS OF THE PODOCYST CUTICLE OF THE SCYPHOZOAN MEDUSA, *CHRYSAORA QUINQUECIRRHA*

R. S. BLANQUET

Department of Biology, Georgetown University, Washington, D. C. 20007

The ability of certain organisms to encyst has long provided exceptional opportunities to study the ways in which cells respond to adverse changes in their environment. Among different groups, the structure and chemical nature of cyst walls vary greatly and, knowledge regarding their morphological and chemical characteristics is of particular importance with regard to our understanding of the physiological and biochemical processes involved during encystment. As comparatively little is known regarding encystment in marine or estuarine organisms, this study was directed to an investigation of the structural and chemical aspects of the podocyst cuticle of the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha*.

Most Scyphozoans, at some stage in their life cycle, exist as either a free-swimming medusa or as a small, relatively sessile polyp. The polyps remain attached, via the pedal disc, to hard substrata such as rock or shell. In some species, small bits of tissue containing epidermal and mesenchymal cells separate from the pedal disc and become covered by a thin cuticle. These structures are termed podocysts. It is common for a single polyp to form numerous cysts as it slowly moves along the substrate.

Early work suggests that the formation of podocysts enables the organism to withstand, for prolonged periods, conditions which are unfavorable to the polyp (Tcheou-Tai-Chuin, 1930). In an extensive histochemical survey, Chapman (1968) reports that the podocyst cuticle of *Aurelia* is chitinous and tanned by phenolic substances. The occurrence of chitin in the Cnidaria are contained in numerous review articles (Richards, 1951; Rudall, 1955; Forester and Webber, 1960; Kent, 1964; Jeuniaux, 1971). Hydrozoan chitin has been studied by both chemical and physical means. Rajulu and Gowri (1967), using the coenosteum of *Millipora*, report that the chitin isolated contained, in addition to glucosamine, significant amounts of galactose and mannose. Whether these sugars were actually incorporated in the chitin molecule or arose from associated carbohydrate could not be determined. Rudall (1955) observed that the x-ray diffraction patterns obtained from the Hydrozoan chitin were atypical and stated that this chitin may

be significantly different from that found in Arthropods with respect to its orientation and crystallinity. Chitin has also been identified via x-ray diffraction studies from the Anthozoan, *Pocillophora* (Wainwright, 1962).

Since the demonstration of chitin in the podocysts of *Aurelia* is based solely on a positive chitosan test (Chapman, 1968) and since the possibility exists that some cnidarian "chitins" may contain sugars other than n-acetylglucosamine, an analysis of *Chrysaora* podocyst chitin was performed using highly specific enzymatic and chromatographic techniques.

Materials and Methods

Podocysts from the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha,* were obtained by suspending trays of cleaned oyster shells from a pier located on St. John's Creek near Solomons, Maryland, during late summer and fall. These shells provided a natural substrate on which sea nettle larvae could settle and develop into polyps with subsequent podocyst production. Cyst-containing shells could be maintained for long periods of time, at 10° C, in aquaria containing synthetic sea water (Instant Ocean) maintained at 15 p.p.t. Large numbers of podocysts could easily be obtained by removing them with a fine dissecting needle.

Electron microscopy

Cysts collected as described above were prepared for electron microscopy by fixing them for 2 hours, at 4° C, in a 2.5% solution of glutaraldehyde in 0.005 M Sorenson's phosphate buffer, pH 7.4. The cysts were then post-fixed for 1 hour, at 4° C, in a 1% solution of osmium tetroxide made up in the same buffer, dehydrated through a graded series of alcohols, and infiltrated with a 1 : 1 mixture of Epon and propylene oxide overnight. Cysts were then placed in a 3 : 1 mixture of Epon and propylene oxide for 12 hours and finally embedded in Epon according to the procedure of Luft (1961). Ultra-thin sections were cut with a DuPont diamond knife, collected on collodion-coated, copper grids (200 mesh), and stained with saturated aqueous uranyl acetate (Watson, 1958).

Isolation of chitin

Podocysts, 3.0 mg wet wt, were placed in 10% disodiumethylenediaminetetraacetate (EDTA), pH 8.0, for 12 hours to remove any calcium which may have been present in the cuticle. The podocysts were next washed in distilled water and then sequentially extracted at room temperature in 8.0 M urea (24 hours), 0.01 N NaOH (6 hours) and 1.0 N NaOH at 100° C for 6 hours. The insoluble material (chitin) was washed in distilled water until all traces of NaOH were removed. This material was examined under the light microscope to insure that all podocyst contents were removed and was found to contain clear, transparent podocyst cuticles. The cuticles were collected by centrifugation and hydrolyzed in 6 N HCL in sealed vessels at 100° C for 6 hours. The hydrolysate was then evaporated *in vacuuo* over NaOH, dissolved in distilled water and the procedure repeated. The residue was then taken up in 80% ethanol for chromatography.

Chitin determination

The above material (at least 50 μg) was applied in two adjacent positions on Whatman No. 1 paper and run together with known standards via descending paper chromatography. Standards, 1.0 mg/ml, consisted of glucose, galactose, glucosamine, galactosamine, n-acetylglucosamine and n-acetylgalactosamine. Three solvent systems were used: Isopropanol: water (4:1), Propanol: Ethyl Acetate: water (7:1:1), and n-Butanol: Ethanol: Acetic Acid: water (5:4:3:2). Duplicate runs were made with each solvent system, one being stained with alkaline silver nitrate (Trevelyan, Proctor and Harrison, 1950) for the detection of reducing sugars and the other with Elson-Morgan (Hexosamine Reagent) for the detection of amino sugars (Elson and Morgan, 1933). Mobilities were measured with respect to glucose for all chromatograms stained with silver nitrate (Rg values) and with respect to the solvent front for chromatograms stained with Elson-Morgan reagent (Rf values).

Podocyst cuticle was also tested for the presence of chitin by subjecting cuticle, obtained before and after treatment in sodium hydroxide, to a partially purified preparation of chitinase (from *Streptomyces griseus*) obtained from the Nutritional Bio-Chemicals Corp. The n-acetylglucosamine released was determined by the methods of Reissig, Strominger and Leloir (1955). Standard curves were prepared from known n-acetylglucosamine samples at concentrations from 1–10 μg/ml.

Tyrosinase determination

Cyst samples were prepared by removing several hundred cysts and homogenizing them in 1.0 ml of distilled water at 2° C. Particulate matter was removed via centrifugation at 2° C at 5000 r.p.m. for 15 minutes. The clear supernatant was analyzed for protein via the methods of Lowry, Rosenbrough, Farr and Randall (1951) and tested for tyrosinase activity by a modification of the fluorometric method of Adachi and Halprin (1967). Equal amounts of cyst homogenate and a solution of 0.75 mM L-tyrosine, 100 μM L-Dopa, 0.75 mM ascorbic acid in 40 mM phosphate buffer, pH 6.9 were mixed and allowed to incubate at 25° C. At periodic intervals, 0.2 ml of this reaction mixture was added to 1.8 ml of 10 mM phosphate buffer, pH 6.5, containing 0.0025% zinc sulfate. After mixing, 0.4 ml of 0.25% potassium ferricyanide was added. Exactly two minutes later, 0.2 ml of 5.0 N NaOH, containing 2% ascorbic acid, was used to stop the reaction. Samples were read after five minutes on an Aminco Fluoro-Microphotometer using Corning filter No. 5860 as the primary filter and Corning Nos. 3385 and 4305 as secondary filters. Blanks were prepared using distilled water in place of the cyst homogenate. Activity was determined from a curve prepared using standard tyrosinase samples. Tyrosinase (from mushroom) was obtained from the Worthington Biochemical Corp., Freehold, New Jersey.

Results

Structural characteristics

Chrysaora podocysts are low, dome-shaped structures measuring about 0.3–0.5 mm in diameter. They consist of a mass of whitish tissue covered by a thin,

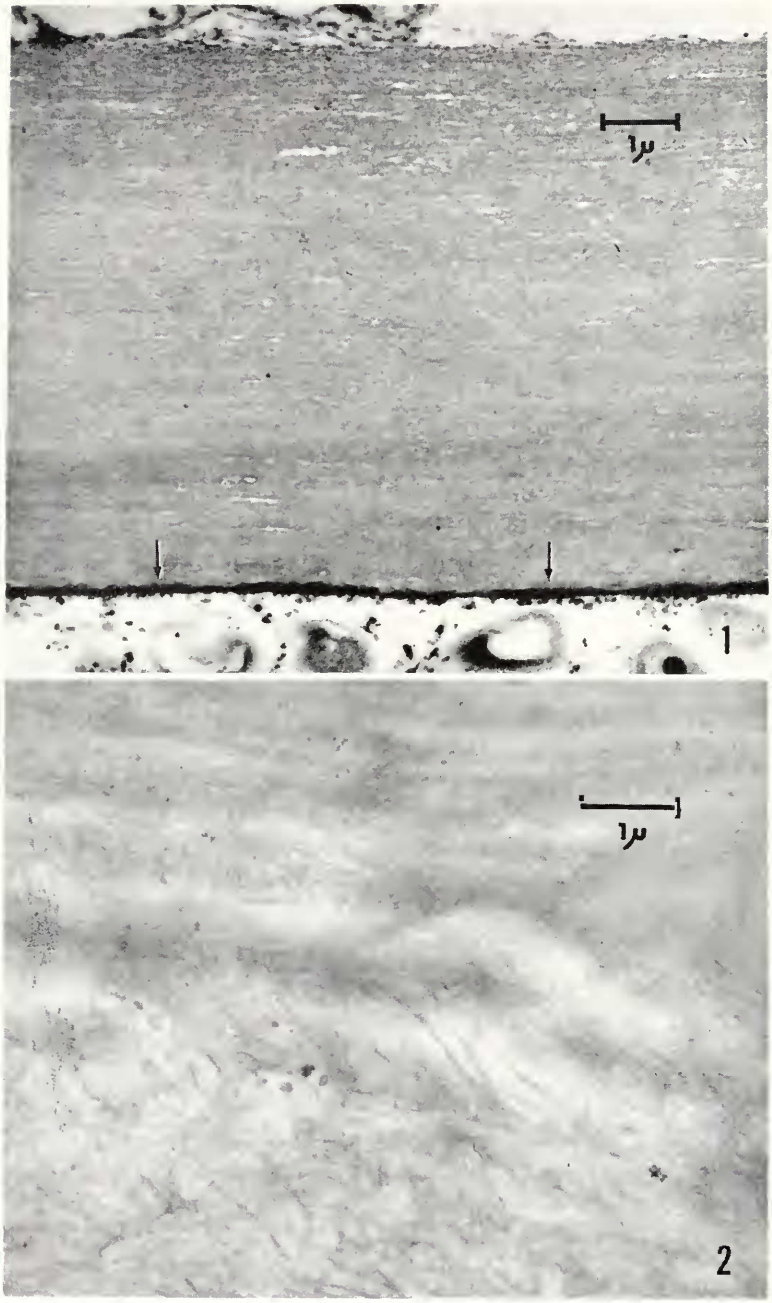


FIGURE 1. A cross section through the cyst wall showing its lamellar structure. Note the darker layer along the surface of cell contact (arrows).

FIGURE 2. An oblique section thru the cyst wall demonstrating the fibrous nature of the lamellae. More compacted lamellae appear at the top of the micrograph.

brown cuticle which varies in thickness from about 9–13 microns. Due to the transparency of the cuticle, large structures, such as nematocysts, can be clearly seen within the intact cyst.

Electron micrographs show this cuticle to be composed of a series of concentric lamellae (Fig. 1). These lamellae appear to be composed of numerous microfibers arranged in sheets (Fig. 2). No structural differentiation can be seen within the cuticle wall except for a narrow layer of varying thickness at the point of contact with the cell mass (Fig. 1, arrow). The cuticular material in this region is heavily stained, appears somewhat granular, and reveals no layered arrangement (Fig. 3).

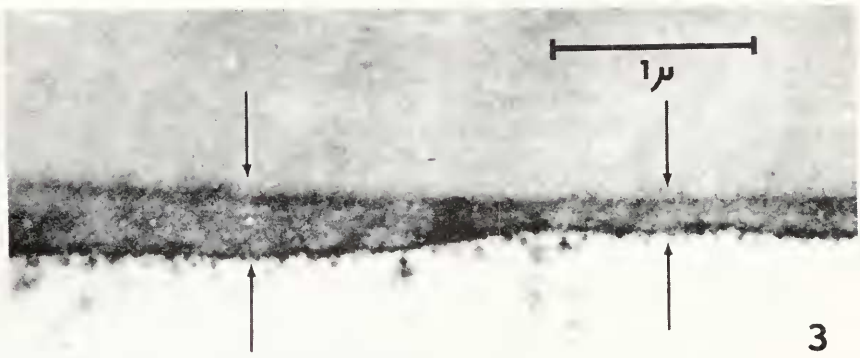
Figure 4 represents a situation in which an outer, thick section of cuticle is separated from a thinner, highly compact section of cuticle by an acellular area of disorganized cuticular lamellae and microfibers. The lamellae at the base of the outer section have separated to a large extent while the lamellae at the periphery are still compact. The inner section of cuticle is highly compact with no trace of lamellar separation. A possible interpretation of these results will be discussed later.

Chemical characteristics

Paper chromatographic analysis of the sugar present in the podocyst cuticle which remained undigested after hot alkali treatment revealed the presence of a single sugar. Comparison of this sugar against known standards in three different solvent systems after development with alkaline silver nitrate or Elson-Morgan reagent showed it to be glucosamine.

Incubation of podocyst cuticle prior to and after hot alkali treatment in the presence of chitinase resulted in the release of N-acetylglucosamine only from cuticles pretreated in hot alkali (Fig. 5). Chitin rarely, if ever, occurs alone in naturally occurring substances but rather as a chitin-protein complex. The necessity for pretreatment of the podocyst cuticle in hot alkali before chitinase degradation could take place indicates that the chitin present in unaltered cuticle is "masked," presumably by its association with protein. This is supported by the tentative identification of several amino acids, *via* paper chromatography, present in the alkali and acid hydrolysates of podocyst cuticles. Due to the extensive degradation of various amino acids during alkali hydrolysis and during acid hydrolysis in the presence of large amounts of sugar, this line of investigation was not pursued.

The possibility that the brownish cuticle of podocysts is the result of a tanning process similar to that which occurs in insect systems was tested by a determination of the polyphenol oxidase (tyrosinase) activity in homogenized cyst extracts. Fluorometric analysis showed an average activity of 0.55 Units/mg protein (Fig. 6). Tanning and hardening of insect cuticle have been shown to be the result of several ortho-dihydroxyphenols present in the cuticle. These phenols are formed by the oxidation of tyrosine by polyphenol oxidase. The resulting polyquinones then crosslink with cuticular protein resulting in a hardening of the cuticle (Locke, 1964; Hackman, 1964).



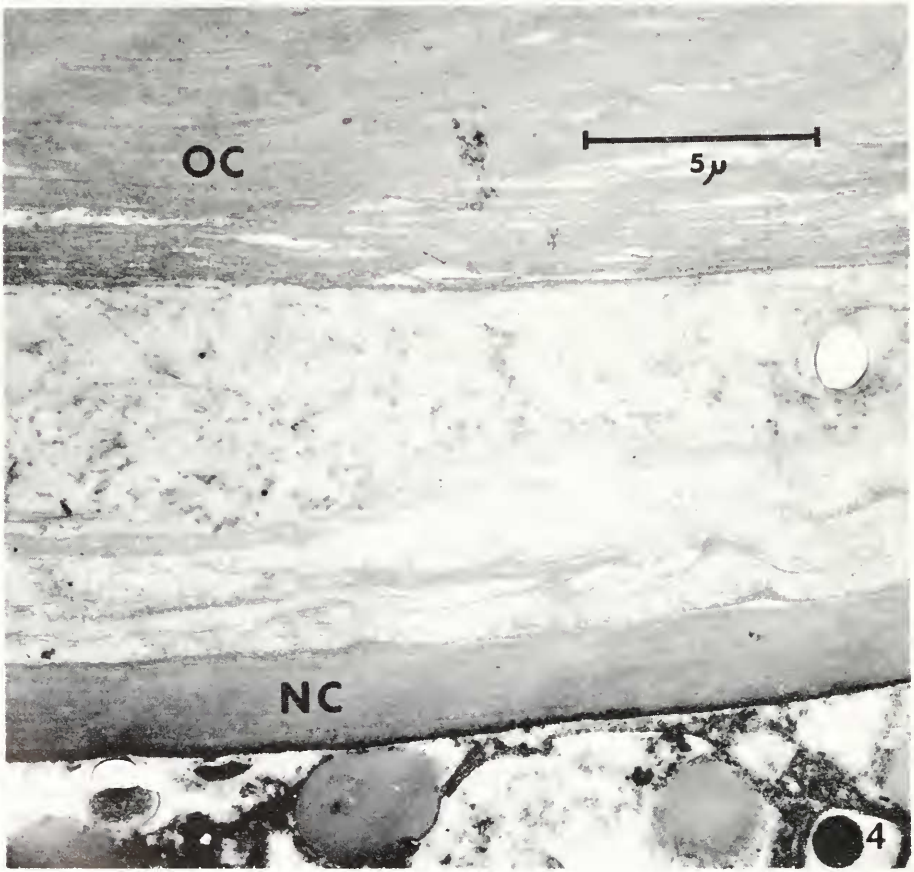


FIGURE 3. A cross section of a cyst wall which has separated from the cellular layer. The darker layer (between the arrows) represents the area of original cell contact. The material composing this layer appears granular and is not arranged into lamella as is the more peripheral material.

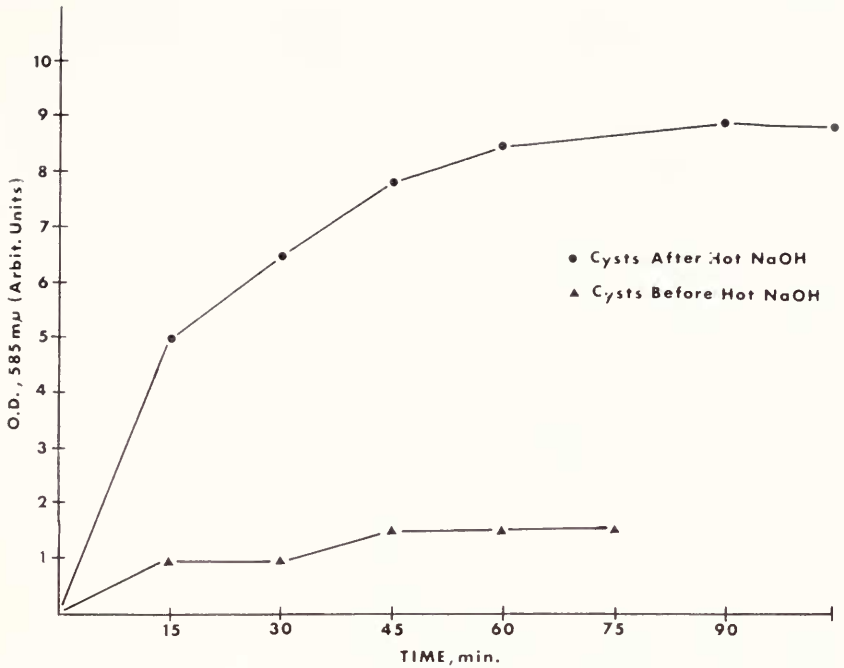


FIGURE 5. The rate of liberation of n-acetylglucosamine from podocyst cuticle in the presence of chitinase prior to (▲) and after (●) cuticle treatment in hot alkali. Spectrophometric determinations were made at 585 mμ.

DISCUSSION

Enzymatic and chromatographic analysis of the polysaccharide isolated from the podocyst cuticle of *Chrysaora* has provided direct evidence that this material is chitin. No sugars, other than n-acetylglucosamine, were detected. Chemically, chitin is a polysaccharide composed of repeating units of n-acetylglucosamine. Hydrolysis occurs only under extreme conditions (*e.g.*, strong mineral acids) and when chitin is complexed with protein, the resulting material is resistant to enzymatic degradation by chitinase and trypsin (Pryor, 1940). Thus, a chitinous cuticle would provide excellent protection against chemical attack. The demonstration that the chitin in *Chrysaora* cuticle is "masked," presumably due to its association with protein, may explain the negative results obtained by Chapman (1968) with chitinase and with various histochemical analyses in an attempt to corroborate the positive chitosan reaction using *Aurelia* podocysts. In the same study, Chapman demonstrated histochemically the presence of polyphenols in the cuticle of *Aurelia* but did not find polyphenol oxidase activity. The extremely sensitive fluorometric analysis employed in this study demonstrated significant activity in *Chrysaora* cysts and provides further evidence that the cuticle is tanned by a process similar to that found in Arthropods (Hackman, 1964).

FIGURE 4. A cross section depicting an old cuticle (OC) separated from a thinner, compact, new cuticle (NC) by an area composed of disrupted lamellae and microfibrils. Note the darker layer at the point of cell contact in the new cuticle and its absence in the old cuticle.

Structurally, cuticular material is arranged as a series of lamellae which, in turn, are composed of microfibers. Such a situation exists in *Aurelia* cuticle (Chapman, 1968) and is similar to the structure of insect exoskeleton (Locke, 1964). It was not possible, however, to distinguish areas within the podocyst cuticle which correspond to the epi-, exo- and endocuticular regions demonstrated in insect and crustacean exoskeletons. Also, there is a lack of pore canals characteristic of these systems.

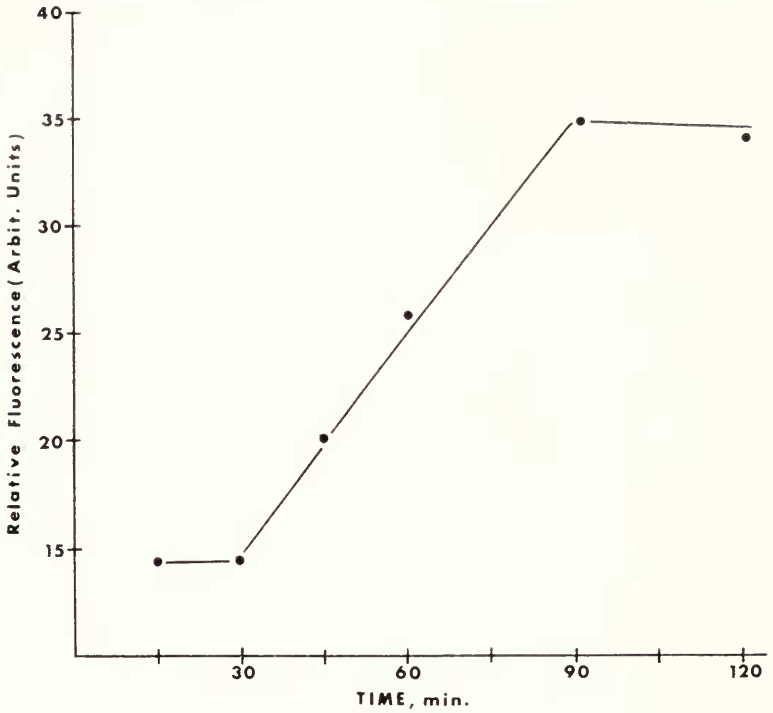


FIGURE 6. Polyphenol oxidase activity of cyst homogenates. The graph represents the rate of conversion of L-tyrosine to L-Dopa in the presence of cyst extracts as measured fluorometrically.

Of particular interest, however, is the fact that, in both insect and cnidarian cuticles, the point of contact with the underlying cells shows different structural characteristics from the immediately overlying more peripheral areas. Schmidt (1956), referred to this area as the subcuticular layer and postulated that it held the insect cuticles to the cells. Locke (1964), however, feels that it is an area of newly secreted endocuticle in which the microfibers are not ordered and have different chemical properties. If such an interpretation could be applied to podocyst cuticle, it would mean that podocyst cells have the continued ability to elaborate new cuticle during encystment. This interpretation is supported by two observations. The first of these regards the situation depicted in Figure 4. In light of what has been shown in Arthropod systems, it is tempting to regard this

micrograph as depicting the shedding of an old cuticle with the simultaneous deposition of a new cuticle. The acellular area between these two cuticles, which shows numerous disorganized microfibers and disrupted lamellae, may represent the partial digestion of the inner layers of the old cuticle. The separation of the inner lamellae and the compactness of the more peripheral lamellae of the old cuticle would support this interpretation. The possibility that at least part of the old cuticle could be reutilized in the formation of the new cuticle is an attractive hypothesis since it conserves the energy store of the encysted cells.

The observation that the homogenates from cysts maintained at 10° C demonstrate significant polyphenol oxidase activity also suggests that the encysted cells possess the ability to manufacture new cuticle. Such an ability would have important survival value since it would enable the organism to repair or replace damaged cuticle during encystment.

I am grateful to Mr. David Cargo, Chesapeake Biological Laboratory, Solomons, Maryland, for his cooperation and the use of his facilities for the collection of the organisms used in this study.

I would also like to thank Mrs. Dorrette Worrell and Mrs. Sandra Zane for expert technical assistance.

Summary

1. The podocyst cuticle of the medusa, *Chrysaora quinquecirrha* is composed of a chitin-protein complex tanned by phenolic substances as demonstrated by chromatographic and enzymatic analysis.

2. Electron micrographs reveal this material to be arranged as concentric layers which, in turn, are composed of microfibers.

3. Evidence is presented which suggests that the encysting cells retain the ability to manufacture new cuticle during encystment.

LITERATURE CITED

Adachi, K., and K. M. Halprin, 1967. A sensitive fluorometric assay method for mammalian tyrosinase. *Biochem. Biophys. Res. Commun.*, **2**: 241–246.

Chapman, D. M., 1968. Structure, histochemistry and formation of the podocyst and cuticle of *Aurelia aurita*. *J. Mar. Biol. Ass. U.K.*, **48**: 187–208.

Elson, L. A., and W. T. J. Morgan, 1933. A colorimetric method for the determination of glucosamine and chondrosamine. *Biochem. J.*, **27**: 1824–1828.

Forster, A. B., and J. M. Webber, 1960. Chitin. Pages 371–393 in M. L. Wolfrom, Ed., *Advances in Carbohydrate Chemistry Vol. 15*. Academic Press, New York.

Hackman, R. H., 1964. Chemistry of the insect cuticle. Pages 471–506 in M. Rockstein, Ed., *The Physiology of Insecta Vol. III*. Academic Press, New York.

Jeuniaux, C., 1971. Chitinous structures. Pages 595–632 in M. Florkin and E. H. Stotz, Eds., *Comprehensive Biochemistry*. Vol. 26c. Elsevier Publishing Co., Amsterdam, London and New York.

Kent, P. W., 1964. Chitin and mucosubstances. Page 93–136 in M. Florkin and H. S. Mason, Eds., *Comparative Biochemistry*. Academic Press, New York.

Locke, M., 1964. The structure and formation of the integument of insects. Pages 379–470 in M. Rockstein, Ed., *The Physiology of Insecta Vol. III*. Academic Press, New York.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. Randall, 1951. Protein measurement with folin-phenol reagent. *J. Biol. Chem.,* **193**: 265–275.

Luft, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.,* **9**: 409–414.

Pryor, M. G. M., 1940. On the hardening of the ootheca of *Blatta orientalis. Proc. Roy. Soc. London Series B,* **128**: 378–393.

Rajulu, G. S., and N. Gowri, 1967. Nature of chitin of the coenosteum of *Millipora* sp. *Indian J. Exp. Biol.,* **5**: 180–181.

Reissig, J. L., J. L. Strominger and L. F. Leloir, 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.,* **217**: 959–966.

Richards, A. G., 1951. *The Integument of Arthropods.* University of Minnesota Press, Minneapolis, 411 pp.

Rudall, K. M., 1955. The distribution of collagen and chitin. *Symposium Soc. Exp. Biol. Number* **IX**: 49–71. (Academic Press, New York and London).

Schmidt, E. L., 1956. The observations on the subcuticular layer in the insect integument. *J. Morphol.,* **99**: 211–231.

Tcheou-Tai-Chuin, 1930. Le cycle evolutif du scyphistome de *Chrysaora. Trav. Sta. Biol. Roscoff,* **8**: 1–179.

Trevelyn, W. E., D. P. Proctor and J. S. Harrison, 1950. Detection of sugars on paper chromatograms. *Nature,* **166**: 444–445.

Wainwright, S. A., 1962. An anthozoan chitin. *Experientia,* **18**: 18–19.

Watson, M. L., 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.,* **4**: 475–478.